Formation of Lipid-linked Sugar Compounds in *Halobacterium salinarium*

PRESUMED INTERMEDIATES IN GLYCOPROTEIN SYNTHESIS*

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The ability of bacitracin to inhibit the growth of *Halobacterium salinarium* suggested that glycosylation of the major envelope component, a high molecular weight glycoprotein, might occur via a pathway involving lipid intermediates. This report demonstrates that the cells have enzymatic activities for formation of lipid-linked sugar compounds having the expected properties of such intermediates.

Whole cell homogenate catalyzed the transfer of sugar from UDP-glucose, GDP-mannose, and UDP-N-acetylglucosamine to endogenous lipid acceptors. Two lipid products were formed from UDP-glucose, two from GDP-mannose, and one from UDP-N-acetylglucosamine. Characterization of the partially purified lipids by ion exchange chromatography, thin layer chromatography, and mild acid and base hydrolysis showed the major properties in each case to have the properties expected for polyisoprenyl phosphoglucose, polyisoprenyl phosphomannose, and polyisoprenyl pyrophospho-N-acetylglucosamine. Estimates of chain length by thin layer chromatography indicate that the lipid has 11 to 12 isoprene units. The N-acetylglucosamine lipid was further purified to homogeneity and analysis confirmed its identity as a C11+polyisoprenyl pyrophospho-N-acetylglucosamine.

The N-acetylglucosamine transferase, present in cell envelope preparations, was partially characterized. The enzyme was found to be extremely halophilic, specifically requiring a high concentration of KCl. Optimum activity was obtained at 4 M KCl and partial substitution of K+ by Na+ resulted in a decrease in activity.

The role of C14-polysoprenyl phosphate- and polysoprenyl pyrophosphate-linked sugars as intermediates in complex polysaccharide synthesis has been demonstrated in a variety of both gram-positive and gram-negative bacteria (1). The initial step in formation of the intermediates is transfer of sugar or sugar 1-phosphate, via membrane-bound enzymes, from nucleoside diphosphosugars to the lipid acceptor, C14-polysoprenyl phosphate. More recently, a role for lipid intermediates in glycosylation of the glycoproteins of eukaryotic cells was suggested (2) and some direct evidence for this has been obtained (3–7). In this case the polysoprenyl phospholipid acceptor has been shown to be a derivative of dolichol, a C25 to C110 alchol having 18 to 22 isoprene units.

The antibiotic bacitracin is thought to kill normal bacteria by interfering with the lipid intermediate cycle of peptidoglycan synthesis (8, 9) and the observation that it inhibits the growth of halobacteria (10) indicated that glycosylation of the *Halobacterium salinarium* cell envelope glycoprotein might occur via a cyclic pathway involving lipid intermediates. The structure of the H. salinarium glycoprotein resembles that of the glycoproteins of eukaryotic cell surfaces with respect to the nature of the carbohydrate-protein linkages, the number, size, and composition of the carbohydrate units, and their localization in the protein (11). These structural similarities and the fact that the glycoprotein is a major envelope component, 50% of the total protein (12), and that it accounts for all of the nonlipid carbohydrate of the cell envelope suggested that *H. salinarium* might provide a useful system for studying the pathway of glycoprotein glycosylation.

**MATERIALS AND METHODS**

GDP-[14C]mannose (160 mCi/mmol), UDP-[14C]galactose (274 mCi/mmol), and UDP-[14C]glucose (250 mCi/mmol) were obtained from New England Nuclear and UDP-[14C]glucuronic acid (290 mCi/mmol) and UDP-N-acetyl[14C]glucosamine (300 mCi/mmol) were obtained from Amersham/Searle. Unlabeled nucleotides and nucleoside diphosphosugars were obtained from Sigma. Farnesyl phosphophosphate[14C]mannose, solanesyl phosphophosphate[14C]mannose, and dolichol phosphate[14C]mannose (13) were kindly given by Josiah F. Wedgwood (Harvard University, Cambridge, Mass.). Synthetic ficaprenyl pyrophosphate-2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-a-D-glucosamine was kindly given by Christopher D. Warren (Harvard Medical School and Massachusetts General Hospital). This compound was converted to ficaprenyl pyrophosphate-N-acetylglucosamine by the method described by Warren et al. (14) for use as a standard in thin layer chromatography. All chemicals and solvents used were reagent grade.

*Halobacterium salinarium*, strain 1 (ATCC 19700), was grown and harvested as previously described (12). Cell homogenates were prepared by suspending washed cell pellets at a 1:1 (wt:v) ratio in 0.1 M...
Formation of Lipid-linked Sugar Compounds in H. salinarium

Tris buffer, pH 8.2, containing 3 M KCl, 1 M NaCl, and 40 mM MgSO₄ and passing once through a French pressure cell (Aminco) at 6000 to 6000 psi. Cell breakage was complete as judged by microscopic examination and resulted in a suspension having a protein concentration of 82 mg/ml. Cell envelopes were prepared as previously described (19).

Assay for incorporation of sugar into lipid products was done by drying the nucleoside diphosphosugar substrate in the assay tube, adding an aliquot of homogenate, or cell envelope suspension (1:1, wet weight-volume) in 0.1 M Tris, pH 8.2, containing 3 M KCl, 1 M NaCl, and 40 mM MgSO₄, mixing, and incubating. Standard incubation conditions were 45 min at 37°C. At the end of the incubation time tubes were placed in a boiling water bath for 3 min to stop the reaction, cooled to room temperature, and the mixtures were extracted twice with two volumes of 1-butanol:0.5 M pyridinium acetate, pH 4.2. Further extraction gave no additional sugar-containing lipid. The combined organic phases were washed twice with water to remove pyridinium acetate and the 1-butanol phase dried in scintillation vials. One drop of bromine was added to decolorize the carotenoids and the excess bromine removed by drying in a stream of air. The samples were then dissolved and counted in toluene/Omnifluor (New England Nuclear).

Degradation of nucleoside diphosphosugar substrates was measured by addition of 10 volumes of cold, 10% trichloroacetic acid to the reaction mixture following lipid extraction. The resulting precipitate was washed twice with cold, 10% trichloroacetic acid, once with ethanol, and once with ethyl ether. This procedure resulted in precipitation of 90 to 100% of the nucleoside diphosphosugar substrate being degraded. When this was examined as described under "Materials and Methods" it was found that the nucleoside diphosphosugars were rapidly destroyed; incubation for 45 min at 37°C results in degradation of 85 to 95% of the substrate. Attempts to inhibit the pyrophosphatase activity (described in more detail below) have been unsuccessful. Despite this problem it was possible to obtain sufficient quantities of the lipid-linked sugar compounds for further characterization.

DEAE-cellulose chromatography of ¹³C-Lipids—The ¹³C-lipids prepared by large scale incubation of whole cell homogenate with GDP-[¹³C]mannose or UDP-[¹³C]glucose were partially purified by DEAE-cellulose chromatography in methanol. Two ¹³C glucolipids (Fig. 1A) and two ¹³C mannolipids (Fig. 1B) were found. In both cases one of them (glucolipid I and mannolipid I) did not bind to DEAE cellulose. Structural glucolipids containing neutral hexoses are present in the membranes of the Halobacteria (12, 17, 18) and synthesis of small amounts of these may account for the unbound, labeled products. Glucolipid II and mannolipid II bound and eluted at approximately 0.70 M NH₄ acetate in a gradient of 0 to 0.1 M NH₄ acetate in methanol, the position expected for glycosyl polyisoprenyl phosphates (19).

DEAE-cellulose chromatography of the ¹³C-lipid formed by incubation of UDP-N-acetyl [¹³C] glucosamine with cell envelopes yielded a single lipid product (Fig. 1C). All of the radioactivity eluted at a single peak at approximately 0.15 M NH₄ acetate in a gradient of 0 to 0.5 M NH₄ acetate in methanol, the position at which glycosyl polyisoprenyl phosphates elute (19).

The fractions containing the partially purified ¹³C-lipid products were pooled and washed to remove NH₄ acetate (as described in the legend to Fig. 1) and further characterized.

Mild Alkaline and Acid Hydrolysis—The partially purified ¹³C-lipids were subjected to mild alkaline methanolysis under conditions sufficient to cause cleavage of fatty acid esters (20). Lipids were dried down, redissolved in 2 ml of 0.1 N KOH in methanol/toluene (2:1), incubated at 0°C for 1 h, neutralized with acetic acid, and 2 ml of chloroform/methanol (1:1) were added. The samples were then dried down, redissolved in 1 ml of 0.1 N KOH in methanol and further characterized.

Transfer of sugars from nucleotide diphosphosugars to lipid—Whole cell homogenates of H. salinarium were examined for the ability to transfer sugars from nucleotide diphosphosugars to lipid-extractable and to trichloroacetic acid-precipitable products. Nucleoside diphosphosugars of all of the sugars known to occur in the envelope glycoprotein (11) were tested. Whole cell homogenates had activities for transfer of mannose, glucose, and N-acetylglucosamine into lipid while cell envelopes had activity for transfer of only N-acetylglucosamine into lipid (Table I). The N-acetylglucosamine transferase activity of cell envelopes was greater than that of whole cell homogenates, probably as a result of both partial purification of the enzyme and of loss of some of the pyrophosphatase activity (see below) upon removal of the supernatant fraction containing the soluble proteins of the cells. The mannose and glucose transferase activities may be loosely bound membrane enzymes which are lost during the preparation of cell envelopes. Their localization was not investigated further. Neither enzyme preparation had activity for transfer of sugars to trichloroacetic acid-precipitable products. If glycosylation of the glycoprotein occurs via lipid intermediates, it is not unlikely that transfer from the lipid to the glycoprotein occurs only after complete carbohydrate units have been assembled. This may explain the failure to observe incorporation of sugars into the glycoprotein under these assay conditions.

The amount of sugar incorporated into lipid was very low, approximately 1 to 2%, suggesting that the nucleoside diphosphosugar substrate was being degraded. When this was examined as described under "Materials and Methods" it was found that the nucleoside diphosphosugars were rapidly destroyed; incubation for 45 min at 37°C results in degradation of 85 to 95% of the substrate. Attempts to inhibit the pyrophosphatase activity (described in more detail below) have been unsuccessful. Despite this problem it was possible to obtain sufficient quantities of the lipid-linked sugar compounds for further characterization.

TABLE I

<table>
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<tr>
<th>Substrate</th>
<th>Whole cell homogenate</th>
<th>Cell envelope</th>
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<tbody>
<tr>
<td>GDP-[¹³C]mannose</td>
<td>11.24</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>UDP-[¹³C]glucose</td>
<td>2.25</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>UDP-[¹³C]galactose</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>UDP-N-acetyl[¹³C]glucosamine</td>
<td>2.4</td>
<td>340</td>
</tr>
<tr>
<td>UDP-[¹³C]glucuronic acid</td>
<td>&lt;0.2</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>
Formation of Lipid-linked Sugar Compounds in H. salinarium

added. Following centrifugation the radioactivity in the aqueous and organic phases was measured. In every case, all (>95%) of the radioactivity was present in the organic phase, indicating that all of the 14C-lipids were stable to this treatment.

Glucolipid I and mannolipid I were stable to mild acid hydrolysis in 0.1 N HCl in chloroform/methanol (2:1) at 23°C (Fig. 2). Glucolipid II, mannolipid II, and the N-acetylglucosamine lipid were labile under these conditions (Fig. 2) and had kinetics of hydrolysis expected if the sugars are linked to the lipid moiety by a glycosidic bond to a phosphate or pyrophosphate (21).

Thin Layer Chromatography—Thin layer chromatography of polisoprenyl phosphosugars on silica gel in Solvent A has been shown to result in separation on the basis of the chain length of the lipid (22). The log of the number of isoprene units in the lipid is linearly related to its chromatographic mobility. Glucolipid II and mannolipid II were examined by this method using polisoprenyl phosphomannose standards of varying lipid chain length (Fig. 3). Both lipid-linked sugar compounds had chromatographic mobilities which indicate that the lipid moieties have 11 to 12 isoprene units.

Fig. 1. DEAE-cellulose chromatography of 14C-lipids. A, 14C-glucolipids; B, 14C-mannolipids; C, N-acetyl 14C glucosamine lipid. Lipids were prepared and extracted as described under "Materials and Methods." The crude lipid extracts in 1-butanol were then dried, redissolved in 10 ml of methanol, and applied to a DEAE-cellulose column (2.5 x 15 cm) equilibrated in methanol. Elution was done with 250 ml of methanol followed by a 0 to 0.1 M NH4 acetate gradient in methanol (300 ml) and finally a 0.1 to 0.5 M NH4 acetate gradient in methanol (600 ml). Fractions of 10 ml each were collected and aliquots assayed for radioactivity in toluene/Omnifluor. Recovery of radioactivity was 95 to 100%. NH4 acetate was extracted from fractions containing the separated 14C-lipids by adding 0.5 volume of H2O and 1.5 volumes of chloroform. The lower phase was washed once with methanol:water (1:1), evaporated to dryness, and finally redissolved in methanol.

Fig. 2 (left). Mild acid hydrolysis of DEAE-cellulose-purified 14C-lipids. Partially purified 14C-lipids were dried, redissolved in 3 ml of 0.1 N HCl in chloroform:methanol (2:1), and incubated at 23°C (23). Aliquots (0.5 ml) were removed at varying times, neutralized with 0.1 ml of 0.5 N NaOH, centrifuged to separate the aqueous and organic phases, the lower phase washed once with methanol:water, and the radioactivity in this phase counted in toluene/Omnifluor.

Fig. 3 (right). Estimated chain lengths of 14C-glucolipid II and 14C-mannolipid II. DEAE-cellulose-purified lipids (Fig. 2) were chromatographed on silica gel in Solvent A along with isoprenyl phosphomannose standards (see "Materials and Methods") of varying chain lengths. Lipids were detected by autoradiography.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous N-acetyl14C glucosamine lipid</td>
<td>0.12   0.28 0.64</td>
</tr>
<tr>
<td>Synthetic fucoprenyl pyrophospho-N-acetylglucosamine</td>
<td>0.12   0.28 0.64</td>
</tr>
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C_{30-40}-polyisoprenyl phosphomannannase and C_{30-40}-polyisoprenyl phosphoglucose and that the N-acetylglucosamine lipid is C_{30-60}-polyisoprenyl pyrophospho-N-acetylglucosamine. Further evidence for the identity of the N-acetylglucosamine lipid was obtained by large-scale purification and characterization of this lipid.

**Purification and Analysis of N-Acetylglucosamine Lipid**—The partially purified N-acetyl[^14C]glucosamine lipid obtained by DEAE-cellulose chromatography with an NH₄ acetate gradient in methanol (as in Fig. 1) was further purified by running twice on DEAE-cellulose using 0.075 M NH₄ acetate for elution (Fig. 4). Phosphate and radioactivity profiles indicated purity from other phospholipids and thin-layer chromatography in Solvents A, B, and C gave single, coincident spots of radioactivity and I₄ staining.

Mild acid hydrolysis (conditions as in Fig. 2) of the isolated lipid yielded a radioactive product in the aqueous phase which co-chromatographed with N-acetylglucosamine on silica gel in Solvent D. Hydrolysis in 4 N HCl at 105° for 2 h gave a product which eluted coincidentally with glucosamine on the short column of a Beckman 120C amino acid analyzer. Phosphate analysis (1b) showed a ratio of 2.1 to 2.4 phosphates/N-acetylglucosamine (based on the specific activity of the UDP-N-acetyl[^14C]glucosamine used), in good agreement with the expected value of 2.0.

The mass spectrum (direct probe) of the lipid moiety resulting from mild acid hydrolysis of the purified N-acetylglucosamine lipid showed a pattern of peaks of the type expected for isoprenoids (23), fragmentation in units of m/e = 18. Due to the small sample size the molecular ion (m+1) was uncertain. Fragments containing up to 8 isoprene units were clearly present and the presence of larger fragments was indicated. The results confirm the isoprenoid nature of the N-acetylglucosamine lipid and are consistent with an estimated chain length of C_{30-60} as indicated by co-chromatography with ficaprenyl pyrophospho-N-acetylglucosamine.

**Properties of N-Acetylglucosamine Transferase**—The properties of the N-acetylglucosamine transferase were studied using isolated cell envelopes. The time course for incorporation from UDP-N-acetyl[^14C]glucosamine into lipid is shown in Fig. 5. Essentially identical results were obtained with substrate concentrations ranging from 0.5 to 1 mM. A determination of the kinetic parameters of the enzyme was not possible due to the pyrophosphatase activity present in the envelope preparations (Fig. 5). Attempts were made to inhibit this activity by addition of alternate substrates or substrate analogues. Nucleoside triphosphates caused inhibition of incorporation, probably as a result of their rapid degradation to nucleoside monophosphates which inhibit the transferase (see below). The nucleoside triphosphate analogues α,β-methylene adenosine 5'-triphosphate and β,γ-methylene adenosine 5'-triphosphate (Sigma) had little or no effect on lipid formation. Sodium pyrophosphate inhibited lipid formation but the methylene analogue, methylene diphosphonic acid, caused stimulation at concentrations from 1 to 20 mM (when added with equal amounts of Mg^{2+}). Maximum stimulation, approximately 2-fold, occurred at 12.5 mM. Concentrations higher than 20 mM inhibited lipid formation, probably by direct inhibition of the transferase. Thus, while methylene diphosphonic acid caused some decrease in the pyrophosphatase activity we were unable to selectively inhibit this activity while retaining transferase activity.

The N-acetylglucosamine transferase activity requires divalent cations. Cell envelope preparations contain Mg^{2+} and are therefore active in the absence of added Mg^{2+} but addition of ethylenediaminetetraacetic acid (60 mM) completely inhibits incorporation into lipid. The pH optimum for formation of the N-acetylglucosamine lipid is approximately 8.2. Nucleoside monophosphates inhibit the incorporation into lipid. UMP is most effective, a concentration of 4 mM giving 50% inhibition (as measured by total incorporation at the end of 45 min).

All Halobacteria enzymes which have been studied require
The N-acetylglucosamine transferase was found to have a specific requirement for a high K+ concentration (Fig. 6A). Activity was not retained if K+ ions were replaced by Na+ (Fig. 6B). The K+ concentration is high inside growing cells (26) and a specific requirement for this cation has been observed for other halophilic enzymes (24). The actual requirement for a high K+ concentration is probably even more pronounced than is indicated by the data shown in Fig. 6 as it was found that the degradation of the UDP-N-acetylglucosamine substrate also required K+ and occurred most rapidly in 4 M KCl (data not shown).

A variety of nonionic detergents of different HLB numbers were tested for their ability to stimulate activity of the N-acetylglucosamine transferase. Those having HLB numbers in the range normally found effective for stimulation of membrane-bound enzymes (27) had either no effect or were inhibitory at concentrations from 0.05 to 0.5%. Brij 99 (HLB = 15.3) was found to be the most effective detergent, but gave only low and variable stimulation (40 to 60% increase in activity). Attempts to stimulate incorporation of N-acetylglucosamine into lipid by addition of fucaprenyl phosphate and detergent were unsuccessful, possibly due to the ineffectiveness of the detergents in solubilizing the lipid at the high salt concentrations necessary for enzymatic activity.

The amount of N-acetylglucosamine lipid formed increases with increasing concentration of UDP-N-acetylglucosamine up to approximately 1.2 mM. At this level approximately 0.6 nmol of lipid are formed/mg of protein in the reaction mixture (Fig. 7). This value allows a minimum estimate to be made of 6 mg of endogenous acceptor lipid/kg of wet cells. By comparison, Streptococcus faecalis has 12.8 mg endogenous acceptor lipid/kg of wet cells based on the yield of isolated polyisoprenol phosphates (20).

**DISCUSSION**

The properties of glucolipid II, mannolipid II, and the N-acetylglucosamine lipid formed by transfer of sugars from nucleoside diphospho sugars to endogenous lipid of Halobacterium salinarium are consistent with their being polyisoprenyl phosphoglucose, polyisoprenyl phosphomannose, and polyisoprenyl pyrophospho-N-acetylglucosamine. Estimates based on mobility on thin layer chromatography indicated a chain length of 11 to 12 isoprene residues. The identity of the N-acetylglucosamine lipid was confirmed by purification and further characterization. Analysis confirmed the presence of two phosphates/glucosamine and the mass spectrum of the lipid confirmed its polysaccharidic nature. Lipid-linked sugar compounds of a similar nature act as intermediates in complex polysaccharide synthesis in prokaryotes (1) and can function in glycosylation of at least some glycoproteins of eukaryotes (2, 5).

The glycoprotein accounts for all of the nonlipid hexose and all of the amino sugar of the H. salinarum cell envelope (12) and it appears likely that the lipid-linked sugars described here function as intermediates in its glycosylation. The glycoprotein contains both N- and O-linked carbohydrate units. All of the amino sugar is present in the N-linked heterosacharide along with mannose, glucose, galactose, uronic acid, and an unidentified amino sugar (11). The presence of an activity for formation of polyisoprenyl pyrophospho-N-acetylglucosamine indicates that this carbohydrate unit is made via lipid-linked intermediates. The other lipid linked sugars described here may also be involved in synthesis of this carbohydrate moiety.

The O-linked units of the glycoprotein consist of di- and trisaccharides of glucosylgalactose and (uronic acid, glucose) galactose and are linked to the protein via galactosylthreonine bonds (11). The failure to detect an activity for formation of a lipid-linked galactose would suggest that synthesis of these units does not occur via lipid-linked intermediates. However, the rapid degradation of the nucleoside diphosphosugar substrate may have prevented detection of the lipid product. We have been unsuccessful in attempting to demonstrate a direct transfer of galactose from UDP-galactose to glycopeptides from which the O-linked units have been removed and preliminary results suggest that bacitracin blocks attachment of both N- and O-linked carbohydrate units to the protein. Synthesis of O-linked carbohydrate moieties of eukaryotic glycoproteins has been demonstrated to occur via both direct transfer and via lipid intermediates. The glucosylgalactose units of collagen are made via soluble glycosyltransferases. These enzymes have

1 Unpublished data.
2 Preliminary results have been published in Ref. 29.
been purified and the possible involvement of lipid intermediates has been excluded (30, 31). In contrast, transfer of mannose from GDP-mannose to threonine/serine in the synthesis of yeast glycoprotein has been shown to occur via a dolichol-linked intermediate (7). Further work is needed to determine which type of pathway is responsible for synthesis of the O-linked carbohydrate units of the H. solinarium glycoprotein.

The failure to detect incorporation of sugars into glycoprotein may have been due to low levels of endogenous acceptor polypeptide. Alternatively, it is not unlikely that transfer of sugars to the glycoprotein occurs only after completed carbohydrate units are assembled on the lipid as is the case in transfer of completed units to growing peptidoglycan chains (1). An obstacle to further study of this aspect of the synthetic pathway is the occurrence of an as yet unidentified amino sugar in the H. solinarium glycoprotein (11).

In the cases where it has been examined, prokaryotes have been found to use C_{10} polyisoprenoids as carrier lipids in complex polysaccharide synthesis. It is of interest to note that H. salinarium appears to use polyisoprenoids of similar chain length (C_{27-44}) in glycoprotein synthesis while eukaryotes use the longer chain length dolichols (C_{10,12}), suggesting that the change in going from prokaryotes to eukaryotes is not a result of the requirements of glycoprotein glycosylation per se. The mechanism of carbohydrate transfer by lipid intermediates is insufficiently understood to allow speculation as to whether the change is a result of an altered requirement for the structure of the carrier lipid or is simply fortuitous. Shorter chain isoprenoids can function as sugar acceptors in in vitro eukaryotic systems (9, 5), but it is not known if they can act as intermediates in glycosylation.

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